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(54) Title: HETEROLOGOUS POLYPEPTIDE EXPRESSION USING LOW MULTIPLICITY OF INFECTION OF VIRUSES

(57) Abstract: The present invention relates to methods and compositions useful for protein expression using low values of multiplicity of infection (MOI).

HETEROLOGOUS POLYPEPTIDE EXPRESSION USING LOW MULTIPLICITY OF INFECTION OF VIRUSES

BACKGROUND OF THE INVENTION

[001] Many therapeutics or potential therapeutics are polypeptides including glycosylated polypeptides, referred to herein as "glycopeptides." The production of a recombinant glycopeptide, as opposed to a recombinant non-glycosylated polypeptide, requires that a recombinantly-produced polypeptide is subjected to additional processing steps, either within the cell or after the polypeptide is produced by the cell, where the processing steps are performed in vitro.

[002] Many expression systems have been developed to produce polypeptides and glycopeptides in eukaryotic or prokaryotic systems. Baculovirus expression system is one that is particularly useful for the production of polypeptides and glycopeptides. This is because baculovirus expression systems have the potential for high-yield expression, and such systems can reliably produce biologically-active peptides, including glycopeptides. A baculovirus expression system requires the infection of insect cells, such as Sf9 cells, with a virus containing one or more cloned nucleic acids of interest. Typically, a high multiplicity of infection (MOI) is needed in order to obtain meaningful and useful levels of expression of a desired polypeptide. However, high MOI levels require a substantial input of time and resources on the front end of a virus expression system. In addition, high MOI levels become exponentially problematic as the scale of the cell culture of interest increases, especially at the industrial level.

[003] There is a need in the art to develop additional methods for production of polypeptides, especially glycopeptides. In particular, there is a need in the art to develop methods that use less virus stock than what is normally required for a virus expression system.

BRIEF SUMMARY OF THE INVENTION

[004] The present invention is based on the discovery that low MOI can be used for polypeptide production in a virus expression system. Accordingly the present invention provides methods of expressing polypeptides in a virus expression system using low MOI values.

[005] In one embodiment, the present invention provides a method of expressing a desired polypeptide in a cell. The method comprises inoculating a cell with a virus, wherein the virus comprises a nucleic acid sequence encoding a desired polypeptide and wherein the inoculation is conducted with a multiplicity of infection (MOI) at less than or equal to 0.00001.

BRIEF DESCRIPTION OF THE DRAWINGS

[006] Figure 1 is a graph illustrating the production of EPO in a baculovirus expression system, as a function of MOI and time.

[007] Figure 2 shows RP-HPLC traces of the positive EPO control.

[008] Figure 3 shows RP-HPLC traces of the positive EPO control.

[009] Figure 4 shows RP-HPLC traces of EPO produced with an MOI of 0.0005.

[0010] Figure 5 shows RP-HPLC traces of EPO produced with an MOI of 0.0005.

[0011] Figure 6 shows RP-HPLC traces of EPO produced with an MOI of 0.005.

[0012] Figure 7 shows RP-HPLC traces of EPO produced with an MOI of 0.005.

DETAILED DESCRIPTION OF THE INVENTION

[0013] The present invention is based on the discovery that low MOI can be used for polypeptide expressions in virus expression systems, especially virus expression systems using insect cells. The present invention therefore provides methods of using low MOI values for expression or production of polypeptides in virus expression systems.

Definitions

[0014] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described herein.

[0015] As used herein, each of the following terms has the meaning associated with it in this section.

[0016] The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

[0017] "Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (i.e., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

[0018] An "isolated nucleic acid" refers to a nucleic acid segment or fragment which has been separated from sequences which flank it in a naturally occurring state, e.g., a DNA fragment which has been removed from the sequences which are normally adjacent to the fragment, e.g., the sequences adjacent to the fragment in a genome in which it naturally occurs. The term also applies to nucleic acids which have been substantially purified from other components which naturally accompany the nucleic acid, e.g., RNA or DNA or proteins, which naturally accompany it in the cell. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., as a cDNA or a genomic or cDNA fragment produced by PCR or restriction enzyme digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

[0019] In the context of the present invention, the following abbreviations for the commonly occurring nucleic acid bases are used. "A" refers to adenosine, "C" refers to cytidine, "G" refers to guanosine, "T" refers to thymidine, and "U" refers to uridine.

[0020] A "polynucleotide" means a single strand or parallel and anti-parallel strands of a nucleic acid. Thus, a polynucleotide may be either a single-stranded or a double-stranded nucleic acid.

[0021] The term "nucleic acid" typically refers to large polynucleotides.

[0022] The term "oligonucleotide" typically refers to short polynucleotides, generally no greater than about 50 nucleotides. It will be understood that when a nucleotide sequence is represented by a DNA sequence (i.e., A, T, G, C), this also includes an RNA sequence (i.e., A, U, G, C) in which "U" replaces "T."

[0023] Conventional notation is used herein to describe polynucleotide sequences: the left-hand end of a single-stranded polynucleotide sequence is the 5' end; the left-hand direction of a double-stranded polynucleotide sequence is referred to as the 5'-direction. A first defined nucleic acid sequence is said to be "immediately adjacent to" a second defined nucleic acid sequence when, for example, the last nucleotide of the first nucleic acid sequence is chemically bonded to the first nucleotide of the second nucleic acid sequence through a phosphodiester bond. Conversely, a first defined nucleic acid sequence is also said to be "immediately adjacent to" a second defined nucleic acid sequence when, for example, the first nucleotide of the first nucleic acid sequence is chemically bonded to the last nucleotide of the second nucleic acid sequence through a phosphodiester bond.

[0024] A first defined polypeptide sequence is said to be "immediately adjacent to" a second defined polypeptide sequence when, for example, the last amino acid of the first polypeptide sequence is chemically bonded to the first amino acid of the second polypeptide sequence through a peptide bond. Conversely, a first defined polypeptide sequence is said to be "immediately adjacent to" a second defined polypeptide sequence when, for example, the first amino acid of the first polypeptide sequence is chemically bonded to the last amino acid of the second polypeptide sequence through a peptide bond.

[0025] The direction of 5' to 3' addition of nucleotides to nascent RNA transcripts is referred to as the transcription direction. The DNA strand having the same sequence as an mRNA is referred to as the "coding strand"; sequences on the DNA strand which are located 5' to a reference point on the DNA are referred to as "upstream sequences"; sequences on the DNA strand which are 3' to a reference point on the DNA are referred to as "downstream sequences."

[0026] Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other

and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns.

[0027] A "recombinant polynucleotide" is one which has been altered or produced by the hand of man. For example, a recombinant polynucleotide may be a polynucleotide isolated from a genome, a cDNA produced by the reverse transcription of an RNA, or an artificial combination of two otherwise separated segments of sequence, e.g., by chemical synthesis or by the manipulation of isolated segments of polynucleotides by genetic engineering techniques.

[0028] "Homologous" as used herein, refers to nucleotide sequence similarity between two regions of the same nucleic acid strand or between regions of two different nucleic acid strands. When a nucleotide residue position in both regions is occupied by the same nucleotide residue, then the regions are homologous at that position. A first region is homologous to a second region if at least one nucleotide residue position of each region is occupied by the same residue. Homology between two regions is expressed in terms of the proportion of nucleotide residue positions of the two regions that are occupied by the same nucleotide residue. By way of example, a region having the nucleotide sequence 5'-ATTGCC-3' and a region having the nucleotide sequence 5'-TATGGC-3' share 50% homology. Preferably, the first region comprises a first portion and the second region comprises a second portion, whereby, at least about 50%, and preferably at least about 75%, at least about 90%, or at least about 95% of the nucleotide residue positions of each of the portions are occupied by the same nucleotide residue. More preferably, all nucleotide residue positions of each of the portions are occupied by the same nucleotide residue.

[0029] As used herein, "homology" is used synonymously with "identity."

[0030] The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, typically exact matches are counted.

[0031] As used herein, the term "promoter/regulatory sequence" means a nucleic acid sequence which is required for expression of a gene product operably linked to the promoter/regulatory sequence. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements which are required for expression of the gene

product. The promoter/regulatory sequence may, for example, be one which expresses the gene product in a tissue specific manner.

[0032] A "constitutive promoter is a promoter which drives expression of a gene to which it is operably linked, in a constant manner in a cell. By way of example, promoters which drive expression of cellular housekeeping genes are considered to be constitutive promoters.

[0033] An "inducible" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a living cell substantially only when an inducer which corresponds to the promoter is present in the cell.

[0034] A "tissue-specific" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a living cell substantially only if the cell is a cell of the tissue type corresponding to the promoter.

[0035] The term "exogenous" as used herein refers to a source other than the composition in question. For example, a plasmid is considered to be derived from an exogenous source when the plasmid is transfected into a cell, *e.g.,* an insect cell, wherein the cell previously did not contain the plasmid. By the term "exogenous nucleic acid" is meant that the nucleic acid has been introduced into a cell or an animal using technology which has been developed for the purpose of facilitating the introduction of a nucleic acid into a cell or an animal.

[0036] "Polypeptide" refers to a polymer composed of amino acid residues, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof linked via peptide bonds. Synthetic polypeptides can be synthesized, for example, using an automated polypeptide synthesizer.

[0037] The term "protein" typically refers to large polypeptides. The term "peptide" typically refers to short polypeptides. However, the term "polypeptide" is used herein to refer to any amino acid polymer comprised of two or more amino acid residues linked via peptide bonds.

[0038] Conventional notation is used herein to portray polypeptide sequences: the left-hand end of a polypeptide sequence is the amino-terminus; the right-hand end of a polypeptide sequence is the carboxyl-terminus.

[0039] As used herein, amino acids are represented by the full name thereof, by the three letter code corresponding thereto, or by the one-letter code corresponding thereto, as indicated in the following table:

Full Name	Three-Letter Code	One-Letter Code
Aspartic Acid	Asp	D
Glutamic Acid	Glu	E
Lysine	Lys	K
Arginine	Arg	R
Histidine	His	H
Tyrosine	Tyr	Y
Cysteine	Cys	C
Asparagine	Asn	N
Glutamine	Gln	Q
Serine	Ser	S
Threonine	Thr	T
Glycine	Gly	G
Alanine	Ala	A
Valine	Val	V
Leucine	Leu	L
Isoleucine	Ile	I
Methionine	Met	M
Proline	Pro	P
Phenylalanine	Phe	F
Tryptophan	Trp	W

[0040] "Mutants," "derivatives," and "variants" of the peptides of the invention (or of the DNA encoding the same) are peptides which may be altered in one or more amino acids (or in one or more base pairs) such that the peptide (or nucleic acid) is not identical to the sequences recited herein, but the peptide (or peptide encoded by the DNA) has the same property as the wild type peptide or peptide of natural sequence.

[0041] A "variant" or "allelic or species variant" of a protein or nucleic acid is meant to refer to a molecule substantially similar in structure and biological activity to either the protein or nucleic acid. Thus, provided that two molecules possess a common activity and may substitute for each other, they are considered variants as that term is

used herein even if the composition or secondary, tertiary, or quaternary structure of one of the molecules is not identical to that found in the other, or if the amino acid or nucleotide sequence is not identical.

[0042] A "recombinant polypeptide" is one which is produced upon expression of a recombinant polynucleotide.

[0043] A "vector" is a composition of matter which comprises an isolated nucleic acid and which can be used to deliver the isolated nucleic acid to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term "vector" includes an autonomously replicating plasmid or a virus. The term should also be construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, polylysine compounds, liposomes, and the like. Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, and the like.

[0044] "Expression vector" refers to a vector comprising a recombinant nucleic acid comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in an in vitro expression system. Expression vectors include all those known in the art, such as cosmids, plasmids (e.g., naked or contained in liposomes) and viruses that incorporate the recombinant nucleic acid.

[0045] As used herein, the term "glycosyltransferase," refers to any enzyme/protein that has the ability to transfer a donor sugar to an acceptor moiety.

[0046] A "glycopeptide" as the term is used herein refers to a peptide having at least one carbohydrate moiety covalently linked thereto. It will be understood that a glycopeptide may be a "therapeutic glycopeptide". The term "glycopeptide" is used interchangeably herein with the terms "glycopolypeptide" and "glycoprotein."

[0047] "Expression" of a polypeptide, as used herein, means any detectable level of transcription and/or translation or activation thereof of a nucleic acid sequence encoding a polypeptide or any detectable level of the activity of a polypeptide.

[0048] An "antibiotic resistance marker" as the term is used herein refers to a sequence of nucleotides that encodes a protein which, when expressed in a living cell, confers to that cell the ability to live and grow in the presence of an antibiotic.

[0049] The term "saccharide" refers in general to any carbohydrate, a chemical entity with the most basic structure of $(CH_2O)_n$. Saccharides vary in complexity, and may also include nucleic acid, amino acid, or virtually any other chemical moiety existing in biological systems.

[0050] "Monosaccharide" refers to a single unit of carbohydrate of a defined identity.

[0051] "Oligosaccharide" refers to a molecule consisting of several units of carbohydrates of defined identity. Typically, saccharide sequences between 2-20 units may be referred to as oligosaccharides.

[0052] "Polysaccharide" refers to a molecule consisting of many units of carbohydrates of defined identity. However, any saccharide of two or more units may correctly be considered a polysaccharide.

[0053] As used herein, a saccharide "donor" is a moiety that can provide a saccharide to a glycosyltransferase so that the glycosyltransferase may transfer the saccharide to a saccharide acceptor. By way of a non-limiting example, a GalNAc donor may be UDP-GalNAc.

[0054] As used herein, a saccharide "acceptor" is a moiety that can accept a saccharide from a saccharide donor. A glycosyltransferase can covalently couple a saccharide to a saccharide acceptor. By way of a non-limiting example, G-CSF may be a GalNAc acceptor, and a GalNAc moiety may be covalently coupled to a GalNAc acceptor by way of a GalNAc-transferase.

Description

Methods

[0055] The present invention provides a method of expressing a desired polypeptide in a cell using a virus expression system. In particular, the method provided by the present invention uses a low multiplicity of infection value, *e.g.,* lower than the values normally used by skilled artisans in the field to inoculate cells for expression of desired polypeptides. In general, the value of multiplicity of infection or MOI represents the ratio between virus and cells to be infected by the virus, *e.g.,* number of

plague forming particles (pfu) per cell or per ml of cell culture. According to the present invention, a low MOI value is less than or equal to 0.00001 (10^{-5}) pfu/cell. In one embodiment, the low MOI value of the present invention is between 0.000001(10^{-6}) to 0.00001(10^{-5}). In another embodiment, the low MOI value of the present invention is between 0.0000001(10^{-7}) to 0.000001(10^{-6}) or 0.0000001(10^{-7}) to 0.00001(10^{-5}). In yet another embodiment, the low MOI value of the present invention is between 0.00000001(10^{-8}) to 0.0000001(10^{-7}), 0.00000001(10^{-8}) to 0.000001(10^{-6}), or 0.00000001(10^{-8}) to 0.00001(10^{-5}).

[0056] It is well within the ability of one skilled artisan to determine the preferred low MOI values or the preferred range of low MOI values for each type or class of polypeptides with respect to each virus expression system. For example, as illustrated in the Experimental Examples section, a cell culture such as an insect cell culture can be infected at a series of MOI values with a baculovirus expression vector containing a nucleic acid sequence encoding a desired polypeptide. By assessing the level of expressed polypeptide at one or more particular time-points for each MOI value, one can identify low MOI values or ranges of low MOI values that are suitable or preferred for expressing the desired polypeptide in the virus expression system.

[0057] Numerous and well-known methods for assaying the expression of a desired polypeptide are available to the skilled artisan. Means for assaying the level of expression of a desired polypeptide include, but are not limited to, enzyme activity assays, protein concentration assays, chromatography, spectroscopy, gel electrophoresis, mass spectrometry, nuclear magnetic resonance, electron paramagnetic resonance, elemental analysis, gas chromatography, differential scanning calorimetry, radioassays, immunoassays, and biological activity assays. Selection of specific methods for monitoring or characterizing expression of a desired polypeptide is based on various factors, including without any limitation, the nature, size, identity, stability, etc. of the desired polypeptide.

[0058] Methods for assaying the biological activity of a desired polypeptide are also available to skilled artisans in the field. For example, the Krystal assay (Krystal, 1983, Exp. Hematol. 11:649-660) can be employed to determine the activity of EPO prepared according to the methods of the present invention.

[0059] Briefly, the assay measures the effect of erythropoietin on intact mouse spleen cells. Mice are treated with phenylhydrazine to stimulate production of

erythropoietin-responsive red blood cell progenitor cells. After treatment, the spleens are removed, intact spleen cells are isolated and incubated with various amounts of wild-type erythropoietin or the erythropoietin proteins described herein. After an overnight incubation, ^{3}H-thymidine is added and its incorporation into cellular DNA is measured. The amount of ^{3}H-thymidine incorporation is indicative of erythropoietin-stimulated production of red blood cells via interaction of erythropoietin with its cellular receptor. The concentration of the erythropoietin protein of the present invention, as well as the concentration of wild-type erythropoietin, is quantified by competitive radioimmunoassay methods well known in the art. Specific activities are calculated as international units measured in the Krystal assay divided by micrograms as measured as immunoprecipitable protein by radioimmunoassay.

[0060] In another example, methods of assessing glycosyltransferase activity are well-known in the art. Various assays for detecting glycosyltransferases have been published. The following are illustrative, but should not be considered limiting, of those assays useful for detecting glycosyltransferase activity. Furukawa et al (1985, Biochem. J., 227:573-582) describe a borate-impregnated paper electrophoresis assay and a fluorescence assay. Roth et al (1983, Exp'l Cell Research 143:217-225) describe application of the borate assay to glucuronyl transferases, previously assayed calorimetrically. Benau et al (1990, J. Histochem. Cytochem., 38:23-30) describe a histochemical assay based on the reduction, by NADH, of diazonium salts. See also U.S. Patent No. 6,284,493 of Roth, incorporated herein by reference. Furthermore, PCR and antibodies can be used to assess levels of expression of desired polypeptides produced by the methods of the present invention.

[0061] According to one feature of the present invention, the low MOI value of the present invention can be used for expression of any desired polypeptide in a virus expression system, *e.g.,* hormones, growth factors, enzymes, inhibitors, receptors including chimeric receptors, cytokines, etc. In one embodiment, desired polypeptides expressed using low MOI value of the present invention include exemplary polypeptides listed in Table 1.

Table 1 Representative examples of polypeptides for low MOI expression

Hormones and Growth Factors • G-CSF • GM-CSF • M-CSF • TPO • EPO • EPO variants • alpha-TNF • Leptin • FSH • HGH • FGH • GLP Enzymes and Inhibitors • t-PA • t-PA variants • Urokinase • Factors VII, VIIa, VIII, IX, X • Dnase • Glucocerebrosidase • Hirudin • α1 antitrypsin • Antithrombin III • α-galactosidase Cytokines and Chimeric Cytokines • Interleukin-1 (IL-1), 1B, 2, 3, 4 • Interferon-alpha (IFN-alpha) • IFN-alpha-2b • IFN-beta • IFN-gamma • IFN-omega • Chimeric diptheria toxin-IL-2	Receptors and Chimeric Receptors • CD4 • Tumor Necrosis Factor (TNF) receptor • Alpha-CD20 • MAb-CD20 • MAb-alpha-CD3 • MAb-TNF receptor • MAb-CD4 • PSGL-1 • MAb-PSGL-1 • Complement • GlyCAM or its chimera • N-CAM or its chimera • Monoclonal Antibodies (Immunoglobulins) • MAb-anti-RSV • MAb-anti-IL-2 receptor • MAb-anti-CEA • MAb-anti-platelet IIb/IIIa receptor • MAb-anti-EGF • MAb-anti-Her-2 receptor

[0062] In particular, a desired polypeptide can be erythropoietin (EPO). Any EPO may be expressed using the methods provided by the present invention, including, but not limited to human EPO. EPO is an acidic glycoprotein of approximately 34 kDa and may occur in three natural forms: alpha, beta, and asialo. The alpha and beta forms differ slightly in carbohydrate components but have the same potency, biological activity and molecular weight. The asialo form is an alpha or beta form with the terminal sialic acid removed.

[0063] As an example, U.S. Patent No. 6,187,564 describes a fusion protein comprising the amino acid sequence of two or more EPO peptides, U.S. Patent Nos. 6,048,971 and 5,614,184 describe mutant EPO molecules having amino acid substitutions at positions 101, 103, 104, and 108. U.S. Patent No. 5,106,954 describes a truncated EPO molecule, and U.S. Patent No. 5,888,772 describes an EPO analog with substitutions at position 33, 139, and 166. U.S. Patents No. 4,703,008, 5,547,933, and 5,621,080 also describe in detail various wild type and mutant EPO molecules. Therefore, the skilled artisan will realize that the present invention encompasses EPO and EPO derivatives and variants as they are well documented in the literature and art as a whole.

[0064] In another embodiment, desired polypeptides suitable for low MOI value in a virus expression system include members of the immunoglobulin family (*e.g.*, antibodies, MHC molecules, T cell receptors, and the like), intercellular receptors (*e.g.*, integrins, receptors for hormones or growth factors and the like) and lectins. Additional examples include renin, clotting factors such as factors V-XII, bombesin, thrombin, hematopoietic growth factor, colony stimulating factors, viral antigens, complement proteins, α1-antitrypsin, erythropoietin, P-selectin glycopeptide ligand-1 (PSGL-1), anti-thrombin III, interleukins, interferons, proteins A and C, fibrinogen, herceptin, leptin, glycosidases, HS-glycoprotein, serum proteins (e.g., α-acid glycoprotein, fetuin, α-fetal protein), β2-glycoprotein, among many others. This list of polypeptides is exemplary, not exclusive.

[0065] In yet another embodiment, a desired polypeptide is a glycosyltransferase, *e.g.*, a galactosyltransferase, fucosyltransferase, glucosyltransferases, N-acetylgalactosaminyltransferases, N-acetylglucosaminyltransferases, glucuronyltransferases, sialyltransferases, mannosyltransferases, glucuronic acid transferases, galacturonic acid transferases, and oligosaccharyltransferases. Suitable glycosyltransferases include those obtained from eukaryotes, as well as from prokaryotes.

[0066] In particular, a desired polypeptide can be a sialytransferase, *e.g.*, sialytransferases listed in Table 2.

Table 2: Exemplary sialyltransferases which use the Galβ1,4GlcNAc sequence as an acceptor substrate

Sialyltransferase	Source	Sequence(s) formed	Ref.
ST6Gal I	Mammalian	NeuAcα2,6Galβ1,4GlCNAc-	1
ST3Gal III	Mammalian	NeuAcα2,3Galβ1,4GlCNAc- NeuAcα2,3Galβ1,3GlCNAc-	1
ST3Gal IV	Mammalian	NeuAcα2,3Galβ1,4GlCNAc- NeuAcα2,3Galβ1,3GlCNAc-	1
ST6Gal II	Mammalian	NeuAcα2,6Galβ1,4GlCNA	
ST6Gal II	photobacterium	NeuAcα2,6Galβ1,4GlCNAc-	2
ST3Gal V	*N. meningitides* *N. gonorrhoeae*	NeuAcα2,3Galβ1,4GlCNAc-	3

1) Goochee *et al., Bio/Technology* **9**: 1347-1355 (1991)

2) Yamamoto *et al., J. Biochem.* **120**: 104-110 (1996)

3) Gilbert *et al., J. Biol. Chem.* **271**: 28271-28276 (1996)

[0067] For example, a desired polypeptide can be a Gal beta-1,3 GalNAc alpha-2,3 sialyltransferase (ST3Gal1), including porcine ST3Gal1, as well as ST3GalI, ST3GalII, ST3GalIII, and ST3GalIV, ST3GalV, and ST3GalVI, ST6GalI, ST6GalII, ST6GalNAcII, ST6GalNAcIII, ST6GalNAcIV, ST8SiaI, ST8SiaII, ST8SiaIII, ST8SiaIV, Siat4, Siat5, Siat6, Siat7, Siat8, Siat10, and Drosophila core1 UDP-galactose:N-acetylgalactosamine-alpha-R beta 1,3-galactosyltransferase (core1 GalT).

[0068] In another example, a desired polypeptide can be a GalNAc alpha-2, 6-sialyltransferase I (ST6GalNAcI). Any ST6GalNAcI polypeptide may be expressed using the methods provided by the present invention, including, but not limited to human ST6GalNAcI, mouse ST6GalNAcI, and chicken ST6GalNAcI. The glycosyltransferase ST6GalNAcI is an essential reagent for glycosylation of therapeutic glycopeptides. Additionally, ST6GalNAcI is an important reagent for research and development of therapeutically important glycopeptides and oligosaccharide therapeutics. ST6GalNAcI is typically isolated and purified from natural sources, or from in vitro and recombinant sources.

[0069] According to another feature of the present invention, the low MOI value of the present invention can be used with any virus expression system, *e.g.,* an expression system using viral infection or viral expression vector to introduce one or more exogenous nucleic acid sequences to a cell to be expressed. For example, the low MOI value of the present invention can be used with any expression system using a virus as an expression vector to infect either eukaryotic or prokaryotic cells or cell culture. In one embodiment, the virus expression system used by the present invention includes infection of a mammalian cell or cell culture with a suitable virus or viral expression vector. In general, mammalian cells or cell culture suitable for viral infection and polypeptide expression include cells or cell lines from humans, monkeys, insects, Drosophila, etc. For example, baculovirus such as *Autographa californica nuclear polyhedrosis virus (*AcNPV) can be used as an expression vector for polypeptide expression in insect cells, *e.g.,* Sf9, Sf21, Drosophila cells, *e.g.,* Schneider S2, or commercially available cell lines, *e.g.,* High Five™ (Invitrogen Life Technologies, Carlsbad, CA).

[0070] Methods of preparing and using virus expression systems are generally known in the art. For example, with respect to baculovirus system, representative references include U.S. Patent No. 5,194,376, U.S. Patent No. 5,147,788, U.S. Patent No. 4,879,236 and Bedard C et al., (1994), Cytotechnology 15:129-138; Hink WT et al., (1991) Biotechnology Progress 7:9-14; Licari P et al., (1992) Biotechnology and Bioengineering 39:614-618, each of which is incorporated herein by reference in its entirety.

Nucleic Acids

[0071] A nucleic acid of the present invention encoding a desired polypeptide may be isolated from numerous sources, including animal or mammalian tissue, insects, nematodes, plants and cDNA libraries. The isolated nucleic acid may be characterized using any technique well-known in the art, as described in detail elsewhere herein. Upon identification of the isolated nucleic acid as encoding a desired polypeptide, the isolated nucleic acid may be modified using well-known molecular biology technology available to the skilled artisan. The invention should not be construed to be limited solely to any one kind nucleic acid encoding any particular desired polypeptide, but rather, should be construed to encompass any

nucleic acid encoding any desired polypeptide that can be expressed recombinantly as described herein. It will be understood, therefore, that a desired polypeptide of the present invention is encoded by a nucleic acid comprising a nucleic acid sequence encoding the desired polypeptide.

Polypeptides

[0072] A desired polypeptide of the invention for expression in a virus expression system with low MOI value should not be construed to be limited in any way. A desired polypeptide of the invention may be any polypeptide, including a polypeptide having a sequence identical to the naturally-occurring form of the polypeptide, a wild type polypeptide, and a polypeptide having one or more amino acid mutations, insertions, deletions, or any combination thereof. Therefore, the present invention encompasses a desired polypeptide that has not yet been identified or expressed recombinantly. The skilled artisan would understand how to prepare, make and use a unique polypeptide in conjunction with the present invention, and the techniques for creating, preparing and using such polypeptides (and related polynucleotides) are well known in the art. For example, see Sambrook et al., 2001, Molecular Cloning: A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory, New York.

[0073] The present invention also provides for analogs of polypeptides. Analogs can differ from naturally occurring polypeptides by conservative amino acid sequence differences or by modifications which do not affect sequence, or by both.

[0074] For example, conservative amino acid changes may be made, which although they alter the primary sequence of the protein or peptide, do not normally alter its function. Conservative amino acid substitutions typically include substitutions within the following groups:

glycine, alanine;
valine, isoleucine, leucine;
aspartic acid, glutamic acid;
asparagine, glutamine;
serine, threonine;
lysine, arginine;
phenylalanine, tyrosine.

[0075] Modifications (which do not normally alter primary sequence) include in vivo, or in vitro chemical derivatization of polypeptides, e.g., acetylation, or carboxylation. Also included are modifications of glycosylation, e.g., those made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing or in further processing steps; e.g., by exposing the polypeptide to enzymes which affect glycosylation, e.g., mammalian glycosylating or deglycosylating enzymes. Also embraced are sequences which have phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine.

[0076] Also included are polypeptides which have been modified using ordinary molecular biological techniques so as to improve their resistance to proteolytic degradation or to optimize solubility properties or to render them more suitable as a therapeutic agent. Analogs of such polypeptides include those containing residues other than naturally occurring L-amino acids, e.g., D-amino acids or non-naturally occurring synthetic amino acids. The peptides of the invention are not limited to products of any of the specific exemplary processes listed herein.

Vectors and Host Cells

[0077] In one embodiment, a desired polypeptide of the invention is contained within a nucleic acid vector, *e.g.,* a viral expression vector suitable for viral infection and polypeptide expression. Such vectors, and techniques for using and making the like, are well-known in the art, and will therefore not be discussed herein in detail. For example, for a review of the production and use of baculovirus-suitable vectors, see Lu et al. (1996, Biotechniques 21:63-68), Luckow et al. (1993, J. Virol. 67:4566-4579), Patel et al. (1992, Nucleic Acids Res. 20:97-104), Peakman et al. (1992, Nucleic Acids Res. 20:495-500), Kitts et al. (1993, Biotechniques 14:810-817), Murphy et al. (1993, Protein Expr. Purif. 4:349-357), Sridhar et al. (1993, Febs Lett. 315:282-286), and Sridhar et al. (1993, Gene 131:261-264), as well as U.S. Patents No. 6,224,882, 6,103,526, 4,879,236, and 4,745,051.

[0078] A desired polypeptide of the present invention, as described above, may be encoded by a nucleic acid, such that the nucleic acid encoding a desired polypeptide is fused to one or more additional nucleic acids encoding a functional polypeptide. By way of a non-limiting example, an affinity tag coding sequence may be inserted into a nucleic acid vector adjacent to, upstream from, or downstream from a desired

polypeptide coding sequence. As will be understood by one of skill in the art, an affinity tag will typically be inserted into a multiple cloning site in frame with a desired polypeptide.

[0079] One of skill in the art will also understand that an affinity tag coding sequence can be used to produce a recombinant fusion protein by concomitantly expressing the affinity tag and desired polypeptide. The expressed fusion protein can then be isolated, purified, or identified by means of the affinity tag. Affinity tags useful in the present invention include, but are not limited to, a maltose binding protein, a histidine tag, a Factor IX tag, a glutathione-S-transferase tag, a FLAG-tag, and a starch binding domain tag. Other tags are well known in the art, and the use of such tags in the present invention would be readily understood by the skilled artisan.

[0080] As will be understood by one of skill in the art, a vector comprising a desired polypeptide of the present invention may be used to express the desired polypeptide as either a non-fusion or as a fusion protein. For example, in a baculovirus expression system, a non-fusion transfer vector may be used as described in Summers, et al., A Manual Of Methods For Baculovirus Vectors And Insect Cell Culture Procedures, Texas Agricultural Experimental Station Bulletin No. 1555 (1987), incorporated herein by reference in its entirety. Such vectors include, but are not limited to pVL941 (BamHI cloning site), pVL1393 (BamHI, SmaI, XbaI, EcoRI, NotI, XmaIII, BglII, and PstI cloning site; Invitrogen; Carlsbad, CA), pVL1392 (BglII, PstI, NotI, XmaIII, EcoRI, XbaI, SmaI, and BamHI cloning site; Invitrogen; Carlsbad, CA), and pBlueBacIII (BamHI, BglII, PstI, NcoI, and HindIII cloning site, with blue/white recombinant screening possible; Invitrogen; Carlsbad, CA). In a baculovirus expression system, a fusion transfer vector may also be used, such as but not limited to pAc700 (BamHI and KpnI cloning site, in which the BamHI recognition site begins with the initiation codon), pAc701 and pAc701 (same as pAc700, with different reading frames), pAc360 (BamHI cloning site 36 base pairs downstream of a polyhedrin initiation codon; Invitrogen; Carlsbad, CA), and pBlueBacHisA, B, C (three different reading frames, with BamHI, BglII, PstI, NcoI, and HindIII cloning site, an N-terminal peptide for ProBond purification, and blue/white recombinant screening of plaques; Invitrogen; Carlsbad, CA).

[0081] Selection of any particular plasmid vector or other DNA vector is not a limiting factor in this invention and a wide plethora of vectors are well-known in the

art. Further, it is well within the skill of the artisan to choose particular promoter/regulatory sequences and operably link those promoter/regulatory sequences to a DNA sequence encoding a desired polypeptide. Such technology is well known in the art and is described, for example, in Sambrook et al. (Third Edition, 2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York), and in Ausubel et al. (1997, Current Protocols in Molecular Biology, John Wiley & Sons, New York).

[0082] The invention thus includes a vector comprising an isolated nucleic acid encoding a desired polypeptide. The incorporation of a desired nucleic acid into a vector and the choice of vectors is well-known in the art as described in, for example, Sambrook et al. (Third Edition, 2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York), and in Ausubel et al. (1997, Current Protocols in Molecular Biology, John Wiley & Sons, New York). In an aspect of the invention, an isolated nucleic acid encoding a desired polypeptide is integrated into the genome of a host cell. In another aspect of the invention, a cell is transiently transfected with an isolated nucleic acid encoding a desired polypeptide. In another aspect of the invention, a cell is stably transfected with an isolated nucleic acid encoding a desired polypeptide.

[0083] The invention also includes cells, viruses, proviruses, and the like, containing such vectors. Methods for producing cells comprising vectors and/or exogenous nucleic acids are well-known in the art. In one embodiment, a nucleic acid encoding a desired polypeptide is introduced into a host cell using a virus expression system. Virus expression systems, such as a baculovirus expression system, are well-known in the art, and will not be described in detail herein. Such virus expression systems are typically commercially available from numerous vendors.

[0084] The skilled artisan will know how to use a host cell-vector expression system for the expression of a desired polypeptide. Appropriate cloning and expression vectors for use with eukaryotic hosts are described by Sambrook, et al., in Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor, N.Y. (2001), the disclosure of which is hereby incorporated in its entirety by reference.

[0085] In one aspect of the invention, a baculovirus, or a baculovirus/insect cell expression system can be used to express a desired polypeptide using a pAcGP67, pFastBac, pMelBac, or pIZ vector and a polyhedrin, p10, or OpIE3 actin promoter. In

another aspect of the invention, a Drosophila expression system can be used with a pMT or pAC5 vector and an MT or Ac5 promoter.

EXPERIMENTAL EXAMPLES

[0086] The invention is now described with reference to the following examples. These examples are provided for the purpose of illustration only and the invention should in no way be construed as being limited to these examples but rather should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Example 1: Titration by Plaque Assay for Erythropietin Producing Baculovirus

Materials and Methods

[0087] Sf-900 II SFM (1X) cells (aqueous) were incubated in a 27°C water bath for at least one hour prior to use. EDTA solutions at pH 8.0 were prepared by adding 10 N NaOH dropwise and filtering the resultant solution through a sterilization filter unit before use. MTT Staining Solution (1 mg/mL) was prepared using Dulbecco's phosphate buffered saline.

Viral titering protocol

[0088] Two Falcon 6-well multi-well tissue culture plates were placed in a biological safety cabinet for each sample to be assayed, as were two plates for each of the controls. Sf9 cells were diluted to 5 X 10^5 cells/mL in a sterile container using pre-warmed Sf-900 II SFM (1X) and mixed gently. The 6-well plates were labeled as follows: Two wells were labeled as "negative control" and two wells were labeled as "positive control," ensuring two empty wells between samples. Duplicate control plates were similarly labeled. For each sample plate, two wells were labeled for every dilution that was assayed. Duplicate sample plates were labeled similarly.

[0089] Sample dilutions were based on the expected baculovirus titer of the sample. For example, a sample with an expected titer of approximately 1 x 10^7 pfu/ml would be assayed at 10^{-6}, 10^{-7} and 10^{-8} dilutions.

[0090] Two mL of diluted Sf9 cells were added to each labeled well and allowed to attach for a minimum of 1 hour at room temperature. Cells were added along the wall of the well, and were gently pipetted to assure cell suspension. Three mL each of Sf-

900 II SFM (1X) were aliquotted into two sterile tubes. Three µl of the positive control viral stock was added to each of the tubes and mixed, to represent a 10^{-3} dilution. For each sample, the appropriate amount of Sf-900 II SFM (1X) was aliquotted into sterile tubes, based on the dilutions that were to be assayed. Samples were prepared in duplicate, and diluted 10-fold so that there was a minimum of 3 ml of each dilution available for assay.

[0091] After one hour incubation of the Sf9 cells, except for the negative control wells, media was removed from the wells and 1 mL each of the viral dilution corresponding to the labeled wells was inoculated. Media was added slowly along the wall of the well to minimize dislodging the cell monolayer, and the resultant mixtures were incubated for at least one hour at room temperature. At the end of one hour, an agar/media solution was prepared by diluting 4% Agar 1:4 with Sf-900 Medium (1.3X).

[0092] The inoculum was removed from each well of the 6-well plate and 2 mL of the agar/media solution was added rapidly by letting the solution run down the side of each well to which it was added. The plates were then incubated for 45 minutes at room temperature. At the end of the 45 minute incubation, paper towels moistened with approximately 2-4 mL of 5mM EDTA were wrapped around the plates. The plates were inverted, placed in a sterile bag and incubated at 27°C for 7-10 days, or until plaques were visible in the positive control wells to the naked eye. After 7-10 days, the plates were unwrapped and 5-7 drops of MTT staining solution was added to each well. Purple color was allowed to develop at room temperature for approximately six hours, or until the entire well was stained purple. Plaques appeared clear against a purple background. Plaques were counted and recorded.

[0093] Analysis of the plaques included a count of the number of plaques present at each dilution. An average was recorded for the duplicate wells at each dilution. The number of plaques between 10-fold dilutions differed by less than a factor of ten. The highest dilution with at least 5 plaques provided a representative number for the plaques formed at that particular dilution.

[0094] The viral titer was then determined as follows. For example, if the well representing the 10^{-8} dilution had 9 colonies, the titer of the viral stock solution is 9×10^8. The units used were Plaque Forming Units/mL (PFU/mL). Therefore, for example, the viral titer is represented as 9×10^8 PFU/mL. If there were not any

plaques present in the negative control and plaques were present in the positive control, then the assay was determined to be valid.

Example 2: Expression of Erythropoietin (EPO) Following Low Multiplicity of Infection of Baculovirus

[0095] In one aspect of the invention, compositions and methods were designed to identify a plaque-purified baculovirus clone for use in the production of EPO, wherein the MOI ranged from low to high values. In one aspect of the invention, the MOI ranged from $1x10^{-3}$ to $1x10^{-6}$. MOI were determined as described elsewhere herein.

[0096] Plaque-purified baculovirus P2 clones containing a nucleic acid sequence encoding human EPO were used in a 2 ml sample volume. Baculovirus Sf 9 cells were obtained at passage #19, wherein the total cell concentration was 2×10^8 in a 50 ml culture volume. AF 900 II serum-free medium was used pre-warmed to 27°C.

[0097] One ml of a P2 clone sample was added to 9 ml of fresh Sf 900 II culture medium and serially diluted five times to obtain viral dilutions of 10^{-3}, 10^{-4} and 10^{-5}. SF 9 cells (45 ml), obtained at passage #19 at 4.5×10^6 cells/ml, were aseptically transferred to a 50 ml conical tube and centrifuged for five minutes at 1000 rpm. The spent medium was removed from each tube and the cells were resuspended in 47 ml of fresh, pre-warmed Sf 900 II medium. Three ml of virus was added to each flask in order to result in MOIs of 10^{-4} to 10^{-6}. Flasks were run in triplicate and centrifuged at 100 RPM, 27 °C, for 120 hours.

[0098]

Table 3. Results of EPO activity assays for baculovirus-expressed EPO P2 clones at varying MOI.

Flask No.	Clone	Estimated MOI	72 hr EPO mg/L	96 hr EPO mg/L	120 hr EPO mg/L
1	C2.1	0.0003	19.40	28.30	8.60
2	C2.1	0.00003	8.60	12.40	38.40
3	C2.1	0.000003	3.50	6.30	17.30
4	C2.3	0.0001	11.10	23.00	50.90
5	C2.3	0.00001	4.50	6.90	32.60
6	C2.3	0.000001	2.50	5.30	7.40
7	C2.4	0.0002	17.50	43.20	23.00
8	C2.4	0.00002	6.70	8.40	33.10
9	C2.4	0.000002	2.20	NA	NA
11	C2.5	0.0001	9.70	25.50	47.10
12	C2.5	0.00001	2.00	14.50	29.10
13	C3.2	0.0006	23.10	50.90	62.20
14	C3.2	0.00006	9.00	13.00	48.00
15	C3.2	0.000006	3.10	10.50	20.20
16	C4.6	0.0005	20.70	46.70	19.90
17	C4.6	0.00005	6.30	16.10	38.40
18	C4.6	0.000005	3.10	8.20	29.30
19	C6.2	0.001	18.50	35.30	53.90
20	C6.2	0.0001	10.50	27.80	33.10
21	C6.2	0.00001	2.70	6.30	19.60
22	C7.3	0.0004	19.70	28.60	22.10
23	C7.3	0.00004	7.60	20.50	43.90
24	C7.3	0.000004	3.30	7.80	7.70
25	C10.4	0.0004	14.90	NA	NA
26	C10.4	0.00004	7.00	11.10	11.60
27	C10.4	0.000004	2.50	9.00	17.70
28	C11.3	0.0005	25.90	25.60	17.40
29	C11.3	0.00005	6.00	11.10	11.80
30	C11.3	0.000005	0.30	9.80	19.10

Example 3: Expression of Erythropoietin (EPO) Following Low Multiplicity of Infection of Baculovirus: Effect of M.O.I. and Time Course

[0099] Sf9 cells (P13; 2.00e8 cells) were resuspended in 50 mL of fresh Sf 900 II SFM medium in each of six 250 mL disposable Erlenmeyer flasks. The cultures were infected with EPO virus stock as described elsewhere herein, at MOI values of 5, 0.5, 0.05, 0.005, 0.0005, and zero (i.e., no virus). Cultures were incubated at 27°C with shaking at 130 rpm (1 inch orbit) and were sampled daily for 5 days. EPO concentration was analyzed by Europium ELISA assay and reverse phase HPLC.

Erythropoietin Europium Based ELISA Protocol

Plate preparation

[00100] Mouse anti-human EPO antibody from Chemicon Cat# MAB1072 is diluted to 2.5 μg/ml in 50 mM Carbonate pH 9.6 coating buffer. This coating antibody solution is dispensed at 100 μl per well in a 96 well plate and is incubated at 4°C overnight.

Blocking

[00101] The plate is washed 3 times with TBS/ 0.2% Tween 20 buffer. Blocking buffer (TBS/ 0.2% Tween 20/ 3% w/v dry milk) is added to each well at 200 μl per well and incubated overnight at 4°C. The plate is washed 3 times with TBS-Tween 0.2% buffer prior to use.

Standard & Test Samples

[00102] A standard curve using Aranesp and pegylated EPO is prepared by 1:2 serial dilution from 25 ng/ml to 0.36 ng/ml in the appropriate sample matrix. A volume of 100 μl is dispensed in duplicate for each standard or each sample. The plate is then incubated over night at 4°C.

Detection

[00103] The plate is washed 3 times with TBS/ 0.2% Tween 20 buffer. Europium labeled mouse anti-human EPO is diluted in PBS (1N) to a concentration of 0.8 μg/ml and 100 μl is dispensed in each well. The plate is incubated at 100 rpm

agitation, room temperature for 1 hour then washed 6 times with TBS/ 0.2%Tween 20 buffer. The enhancement solution (Perkin Elmer cat# CS500-100) is added to each well at 200 µl per well and the fluorescence is read with the Wallac plate reader.

Table 4: EPO produced (µg/ml) as a function of time and MOI.

Time (hours)	Control (ug/mL)	MOI 5 (ug/mL)	MOI 0.5 (ug/mL)	MOI 0.05 (ug/mL)	MOI 0.005 (ug/mL)	MOI 0.0005 (ug/mL)
0	0.0	0.0	0.0	0.0	0.0	0.0
24	0.0	5.7	6.4	5.9	4.1	3.1
48	0.0	8.7	10.7	11.4	15.9	18.4
72	0.0	5.3	11.3	13.3	20.5	19.7
96	0.0	4.1	4.9	16.5	47.4	41.6
120	0.0	2.9	5.2	17.3	14.7	27.9

Table 5: Standard curve data for EPO expression analysis

Study:	Analysis of Epo Samples by RP-HPLC Method		
Sample	Insect Cell EPO	**EPO Extinction Coefficient**	1.24
Ref. Standard*:	Bovine Serum Albumin	**Purity of Std.:**	100%
Ref. Std Conc.	2000 ug/mL		

Std. Concentration (µg/mL) [mass]	**Chrom.#**	**Retention Time (min)**	**Peak Area @A280 (mAU)**
13.9	0012.5ug	10.72	11659
27.8	0025ug	10.47	56413
56.1	0050ug	10.37	101807
112.8	0100ug	10.13	235989
200.4	0200ug	9.87	491248

Table 6: Reverse-phase HPLC analysis of expressed EPO samples.

Samples	Reference #	Retention Time (min)	Peak Area (mAU)	Peak Concentration (µg/mL)	RP-HPLC Total Protein Concentration (µg/mL)
EPO Positive Control (diluted 1:10)	007EPOLot0401077	20.12	154966	39.0	150.5
		20.817	496137	111.5	
MOI = 0.0005 sample, 96 hours	009MOI 00057	20.12	28401	12.1	44.8
		20.85	125111	32.7	
MOI = 0.005 sample, 96 hours	010MOI 0057	20.42	61152	19.1	32.1
		20.883	32397	13.0	
Sf9 culture supernatant + 50 µg/mL EPO Positive Control	011Spike	20.10	31054	12.7	50.9
		20.8	151305	38.2	

Table 7: ELISA vs. Reverse Phase HPLC EPO concentration analysis

Sample	ELISA #1 Concentration (µg/mL)	ELISA #2 Concentration (µg/mL)	ELISA AVG Concentration (µg/mL)	RP-HPLC Concentration (µg/mL)
Epo Positive Control (diluted 1:10)	175.0	139.0	157.0	150.5
MOI = 0.0005 sample, 96 hours	41.6	50.4	46.0	44.8
MOI = 0.005 sample, 96 hours	47.4	47.8	47.6	32.1*
Sf9 culture supe + 50 µg/mL EpoPositive Control	48.4	n/a	48.4	50.9

Example 4: Expression of Human, Mouse and Chicken ST6GalNAc Following Low Multiplicity of Infection of Baculovirus

[00104] Sf 9 cells were used in a culture volume of 50 ml in a 500 ml shake flask. In one aspect of the invention, the target cell concentration used was 3e6 cells/ml, at a target MOI of: 5e-2 to 5e-6. Baculovirus virus titers used included: human ST6GalNAcI clone, 5e5 pfu/ml; mouse ST6GalNAcI clone, 3.75e7 pfu/ml;

and chicken ST6GalNAcI clone, 2.2e7 pfu/ml. The total cells used in this experiment were 150e6 (3e6 per/ml, 50 ml total). The total virus counts used for the highest MOIs included 7.5e6/5e5 for human (5e-2 x 150e6)/ 5e5, in 15 ml), 7.5e6/3.75e7 for mouse (5e-2 x 150e6)/3.75e7, in 0.2 ml), and 7.5e6/2.2e7 for chicken (5e-2 x 150e6)/2.2e7, 0.34 ml).

[00105] Dilution procedures were carried out by adding 15 ml and 0.15 ml of human virus for the first two MOI, respectively. Virus was diluted as follows: 0.3 ml human virus + 9.7 ml Sf 900II SFM for MOI 5e-4, 1.0 ml 5e-4 + 9 ml Sf 900II SFM for MOI 5e-5, 1.0 ml 5e-5 + 9 ml Sf 900II SFM for MOI 5e-7, 1.0 ml 5e-6 + 9 ml Sf 900II SFM for MOI 5e-8, 0.4 ml mouse virus + 9.6 ml Sf 900II SFM for MOI 5e-2, and 0.68 ml human virus + 9.32 ml Sf 900II SFM for MOI 5e-2. Samples prepared for lower MOI were prepared in the same manner as described herein for the samples for the human virus.

[00106] Individual experiments were started by adding 5 ml of each dilution to 45 ml of Sf 9 cells. Cell concentrations were measured and 1 ml sample was withdrawn for an ST6GalNAcI radioactive assay on days 2, 3 and 4.

Table 8. Results of ST6GalNAcI activity assays for baculovirus-expressed human, mouse and chicken ST6GalNAcI clones at varying MOI. The actual starting cell concentration ranged between 1.6e6 to 2.35e6. The cells were in PSG17 and 100% medium exchanged by spun down at 600 g for 3 min.

MOI	ST6GalNAcI Activity	Activity in Unit/L		
		Hours		
		43	67	91
0.05	human	2.239	1.101	2.33
0.005	human	0.553	1.024	2.528
0.0005	human	0	1.293	3.573
0.00005	human	0	0.986	3.274
0.000005	human	0	0.038	3.744
0.05	mouse	0.092	0.32	0.499
0.005	mouse	0	0.87	0.37
0.0005	mouse	0	0.89	0.799
0.00005	mouse	0	0.333	0.844

MOI	ST6GalNAcI Activity	Activity in Unit/L		
		Hours		
0.000005	mouse	0	0.026	1.102
0.05	chicken	2.41	8.063	17.045
0.005	chicken	0	6.137	18.346
0.0005	chicken	0	5.689	20.294
0.00005	chicken	0	2.221	22.356
0.000005	chicken	0	0.166	21.746
*chicken T233JP titer calculated as 2.2e7, actual titer was 7.48e6.				
MOI		Sf9 (e6 cells/ml)		
		Hours		
		43	67	91
0.05	human	1.95	1.95	1.55
0.005	human	2.15	3.8	2.9
0.0005	human	2.3	4.6	4.5
0.00005	human	3.65	6.5	4.5
0.000005	human	2.15	6.85	8
0.05	mouse	1.6	2.4	2.05
0.005	mouse	1.5	2.8	3.67
0.0005	mouse	2.4	3.1	4.5
0.00005	mouse	4	3.6	6.2
0.000005	mouse	2.7	6.4	7.4
0.05	chicken	2.6	1.6	0.8
0.005	chicken	3.75	3.35	1.9
0.0005	chicken	2.6	3.35	1.25
0.00005	chicken	1.8	4.95	3.4
0.000005	chicken	2.45	7.5	3.95

Example 5: Expression of Human ST6GalNAc Following Low Multiplicity of Infection of Baculovirus

[00107] Sf 9 cells were used in a culture volume of 50 ml in 500 ml shake flask. The target cell concentration used was 3e6 cells/ml, and the target MOI used was 5e-5 to 5e-8. Baculovirus titer used for the human ST6GalNAcI clone was 5e5 pfu/ml. Total number cells used was determined to be 150e6 (3e6 per/ml x 50 ml). The total number of virus particles used for the highest MOI used was 7500/5e5 ((5e-5 x 150e6)/ 5e5, in 0.015 ml).

[00108] For use in this experiment, virus samples were diluted as follows: 0.3 ml human virus + 9.7 ml Sf 900II SFM for MOI 5e-3, 1.0 ml 5e-3 + 9 ml Sf 900II SFM for MOI 5e-4, 1.0 ml 5e-4 + 9 ml Sf 900II SFM for MOI 5e-5, 1.0 ml 5e-5 + 9 ml Sf 900II SFM for MOI 5e-6, 1.0 ml 5e-6 + 9 ml Sf 900II SFM for MOI 5e-7, and 1.0 ml 5e-7 + 9 ml Sf 900II SFM for MOI 5e-8.

[00109] Experiments were initiated by adding 5 ml of each virus dilution to 45 ml of Sf 9 cells. Cell concentration was measured and 1 ml of each sample was withdrawn for an ST6GalNAcI radioactive assay on days 3, 4, 5, 6 and 7.

Table 9. Results of ST6GalNAcI activity assays for baculovirus-expressed human ST6GalNAcI clones at varying MOI. The starting cell concentration is 3e6, and the cells were used in PSG13 and 100% medium-exchanged by centrifugation at 600xg for 3 minutes.

MOI		ST6GalNAcI Activity in Unit/L				
		Day				
		3	4	5	6	7
5.00E-05	human	0.836	2.062	3.796	4.671	3.285
5.00E-06	human	0.49	2.973	5.349	5.648	4.04
5.00E-07	human	0.041	2.233	3.599	6.175	5.432
5.00E-08	human	0.008	0.111	0.805	1.155	1.456
MOI		Sf9 (e6 cells/ml)				
		Day				
		3	4	5	6	7

MOI		ST6GalNAcI Activity in Unit/L				
		Day				
5.00E-05	human	6.5	5.9	5.5	2.95	0.9
5.00E-06	human	7.85	6.75	7.55	3.85	1.15
5.00E-07	human	11	12.9	10.1	12.7	6.15
5.00E-08	human	13.1	14.2	9.8	8.8	3.65

[00110] Additional experiments illustrated that, after a 4-day incubation, human ST6GalNAcI activity reached 3.744 U/L in a culture infected at a MOI of 5e-6 (e = base log 10, so 5e-6 is 5×10^{-6}) and that, after a 6-day incubation, ST6GalNacI activities of 6.175 U/L was observed in a culture infected at an MOI of 5e-7. In a larger scale culture of 2 liters, after a 5-day incubation, human ST6GalNAcI activity of 5.099 U/L was observed in a culture infected at an MOI of 5e-6. The large-scale culture results are comparable to those of a 50 ml culture infected at an identical MOI, wherein the ST6GalNAcI activity at day 5 was 5.349 U/L.

Example 6: Expression of G-CSF Following Low Multiplicity of Infection of Baculovirus

[00111] Following general procedures described above, the following tables demonstrate data obtained for G-CSF expression using low MOI values.

Table 10

G – CSF Low MOI Study				
MOI		Activity in Unit/L Hours		
		43	67	91
0.05	Clone 1	2.239	1.101	2.33
0.005	"	0.553	1.024	2.528
0.0005	"	0	1.293	3.573
0.00005	"	0	0.986	3.274
0.000005	"	0	0.038	3.744
0.05	Clone 2	0.092	0.32	0.499
0.005	"	0	0.87	0.37
0.0005	"	0	0.89	0.799
0.00005	"	0	0.333	0.844
0.000005	"	0	0.026	1.102
0.05	Clone 3	2.41	8.063	17.045
0.005	"	0	6.137	18.346
0.0005	"	0	5.689	20.294

0.00005	"	0	2.221	22.356
0.000005	"	0	0.166	21.746

Table 11

G-CSF Low MOI Study						
MOI		Activity in Unit/L				
		Day				
		3	4	5	6	7
5.00E-05	Clone 1	0.836	2.062	3.796	4.671	3.285
5.00E-06	"	0.49	2.973	5.349	5.648	4.04
5.00E-07	"	0.041	2.233	3.599	6.175	5.432
5.00E-08	"	0.008	0.111	0.805	1.155	1.456

Example 7: Endpoint Dilution Baculovirus Titer Assay

[00112] The titer of viral stocks was determined using end point dilution assay. Cells were counted and viability determined as described elsewhere herein. Cells were at least 90% viable and in log phase growth.

[00113] Cells were diluted with fresh media to a density of 2.5e5 cells/mL in 10mL of media, and plated at 10uL/well in a 72-well microtiter plate. Ten wells were plated per row. Media (SFM Sf900 II) was plated alone in the last 2 wells of each row.

[00114] Serial 1:10 dilutions of virus stock were prepared from 1.0 e-1 to 1.0 e-9. Virus stock in a volume of 100uL was diluted into 900uL SFM Sf-900 II media to a final volume of 1.0 mL. The first row (A) was made the negative control row by plating 10μl of media alone after Sf9 cells have been plated. Ten microliters of the 1.0 e-1 dilution tube was plated into each of 10 wells in row B of the first plate. Plating continued until all dilutions were plated. Plates were incubated at 27°C for 7 days in a humid container.

[00115] Plates were examined under a microscope using a 10X objective. All wells were scored as either infected or not infected. Data was entered into the titer determination worksheet described in Table 8 in order to determine virus titer of unknown samples. The Reed-Muench formula (Reed, L.J., and Muench, H. (1938), Amer. Jour. Hygiene, 27, 493-497) was used to determine the 50% infectivity dose ($TCID_{50}$) of virus in order to determine viral titer.

Table 12: Titer determination calculations for endpoint dilution assay.

Viral Dilution	Infected	Uninfected	Total Infected	Total Uninfected	%
No Virus	0	10			

Viral Dilution	Infected	Uninfected	Total Infected	Total Uninfected	%
10^{-1}	10	0	51	0	100
10^{-2}	10	0	41	0	100
10^{-3}	10	0	31	0	100
10^{-4}	10	0	21	0	100
10^{-5}	8	2	11	2	84.62
10^{-6}	3	7	3	9	25
10^{-7}	0	10	0	19	0
10^{-8}	0	10	0	29	0
10^{-9}	0	10	0	39	0
PD= (High%-50)/ (High% - Low%)			0.580645161		
Log TCID50			-5.58064516		
TCID50 (per10 uL = vol. of virus plated)			380,754.6021		
TCID50 (per uL)			38,075.46021		
TCID50 (per mL)			38,075,460.21		
Viral Titer (pfu/mL) = TCID50 (per mL) x 0.69			2.63e7		

10^{-1} to 10^{-4} all infected wells
10^{-5} 8 infected, 2 non-infected
10^{-6} 3 infected, 7 non-infected
10^{-7} to 10^{-9} all non-infected
High% = % infected immediately above 50%
Low% = % infected immediately below 50%
PD = Proportionate Distance of a 50% infectivity response
log $TCID^{50}$ = log of the dilution giving a response greater than 50% minus PD of that response
$TCID^{50}$ = Reciprocal of log TCID50
PFU (plaque forming units) = $TCID^{50}$ x 0.69

[00116] The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety. While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

CLAIMS

What is claimed is:

1. A method of expressing a desired polypeptide in a cell, said method comprising inoculating a cell with a virus, wherein the virus comprises a nucleic acid sequence encoding a desired polypeptide and wherein the inoculation is conducted with a multiplicity of infection (MOI) at less than or equal to 0.00001.

2. The method of claim 1, wherein the MOI is between 0.000001 and 0.00001.

3. The method of claim 1, wherein the MOI is between 0.0000001 and 0.00001.

4. The method of claim 1, wherein the MOI is between 0.00000001 and 0.00001.

5. The method of claim 1, wherein the desired polypeptide is a therapeutic polypeptide.

6. The method of claim 1, wherein the desired polypeptide is selected from the group consisting of a hormone, growth factor, receptor, enzyme, inhibitor, and cytokine.

7. The method of claim 1, wherein the desired polypeptide is a glycosyltransferase.

8. The method of claim 1, wherein the desired polypeptide is selected from the group consisting of a galactosyltransferase, fucosyltransferase, glucosyltransferases, N-acetylgalactosaminyltransferases, N-acetylglucosaminyltransferases, glucuronyltransferases, sialyltransferases, mannosyltransferases, glucuronic acid transferases, galacturonic acid transferases, and oligosaccharyltransferases.

9. The method of claim 1, wherein the cell is a mammalian cell.

10. The method of claim 1, wherein the cell is an insect cell.

11. The method of claim 1, wherein the cell is selected from the group consisting of Sf9, Sf21, High Five™, and S2.

12. The method of claim 1, wherein the virus is a baculovirus.

13. The method of claim 1, wherein the virus is an AcNPV.

14. The method of claim 1, wherein the desired polypeptide is a therapeutic polypeptide and the MOI is between 0.00001 and 0.000001.

15. The method of claim 1, wherein the desired polypeptide is EPO or G-CSF, the virus is baculovirus, the cell is Sf9 and the MOI is between 0.00001 and 0.000001.

16. The method of claim 1, wherein the desired polypeptide is a glycosyltransferase and the MOI is between 0.00001 and 0.000001.

17. The method of claim 1, wherein the desired polypeptide is a glycosyltransferase, the virus is baculovirus, the cell is Sf9 and the MOI is between 0.00001 and 0.000001.

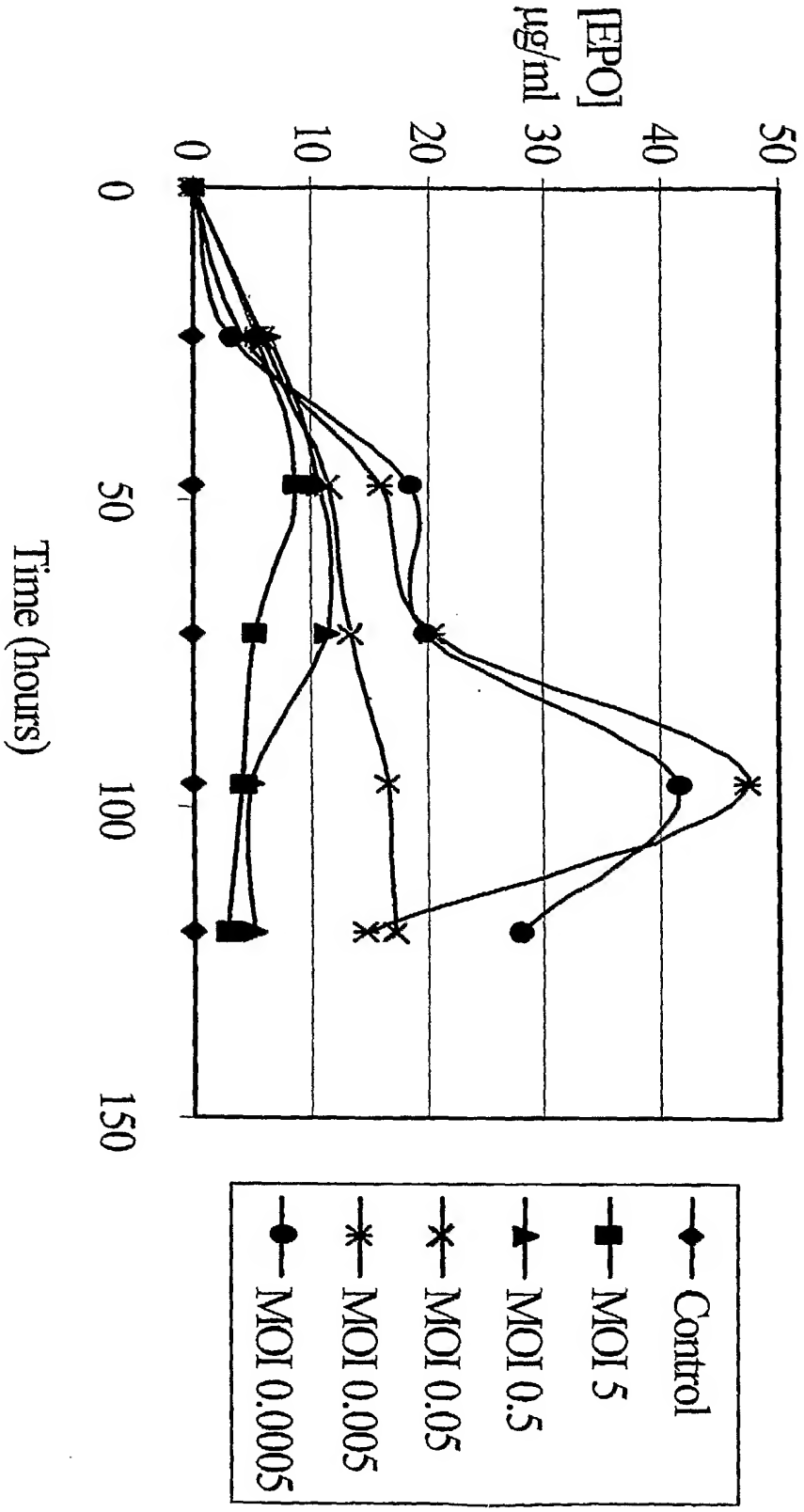
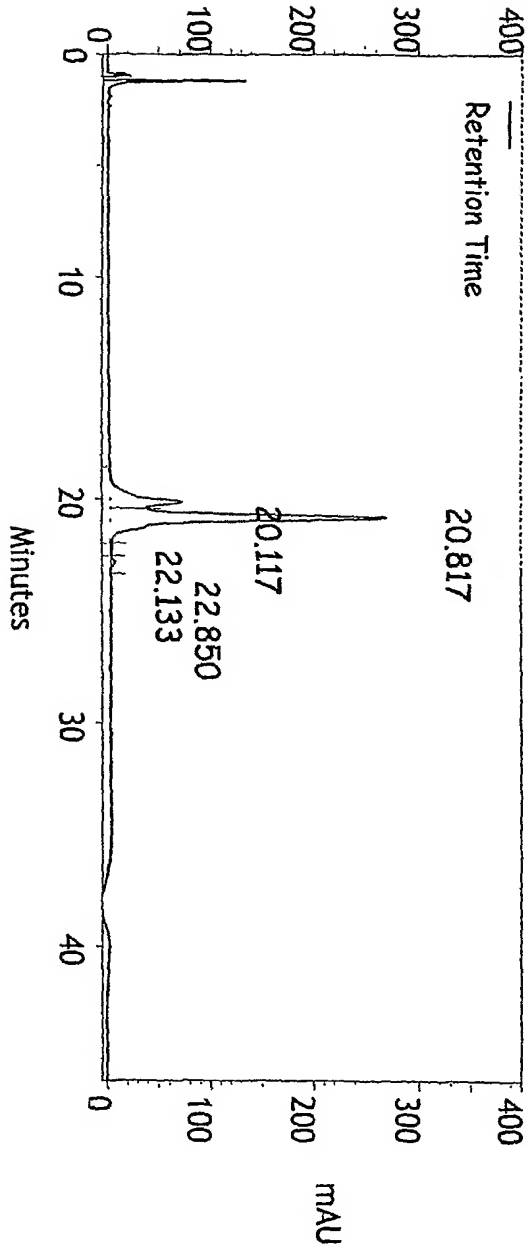


Fig. 1

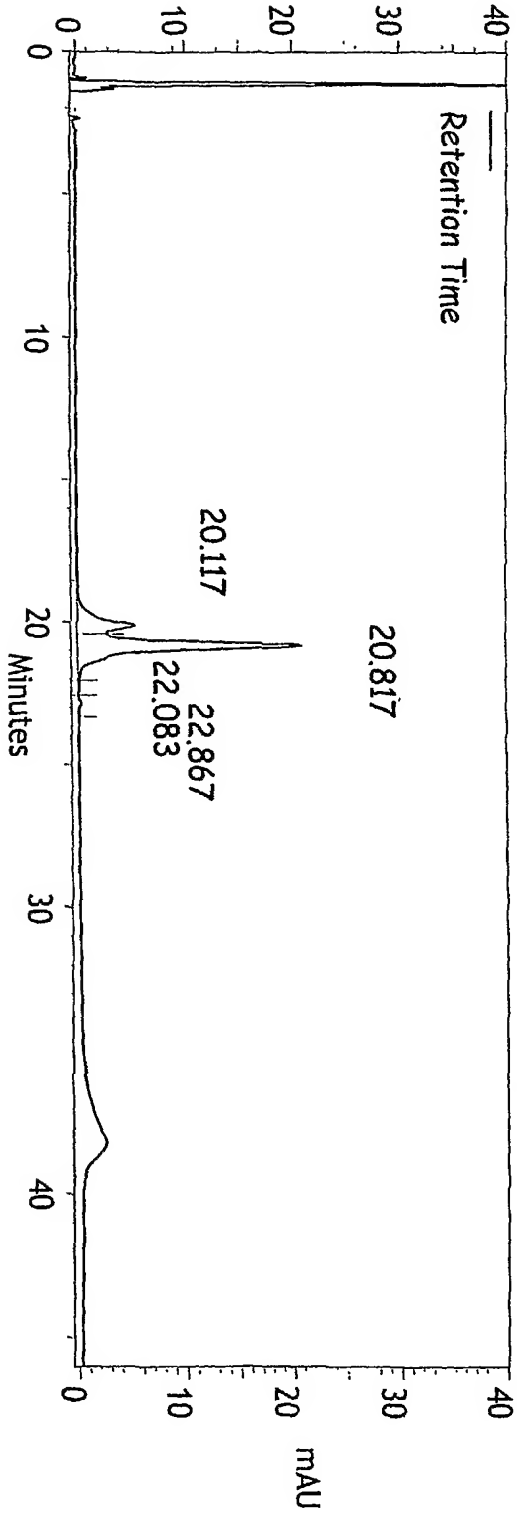
EPO Production

Fig. 2



Retention Time	Area	Area%	Height	Height %
20.117	1982122	23.44	68892	20.24
20.817	6369005	75.30	266182	78.20
22.133	41194	0.49	1703	0.50
22.850	65514	0.77	3605	1.06
Totals	8457835	100.00	340382	100.00

Fig. 3



Retention Time	Area	Area%	Height	Height %
20.117	154966	23.47	5364	20.20
20.817	496137	75.15	20732	78.07
22.083	3463	0.52	160	0.60
22.867	5673	0.86	299	1.13
Totals	660239	100.00	26555	100.00

Calculated [Epo] =150.5 ug/mL

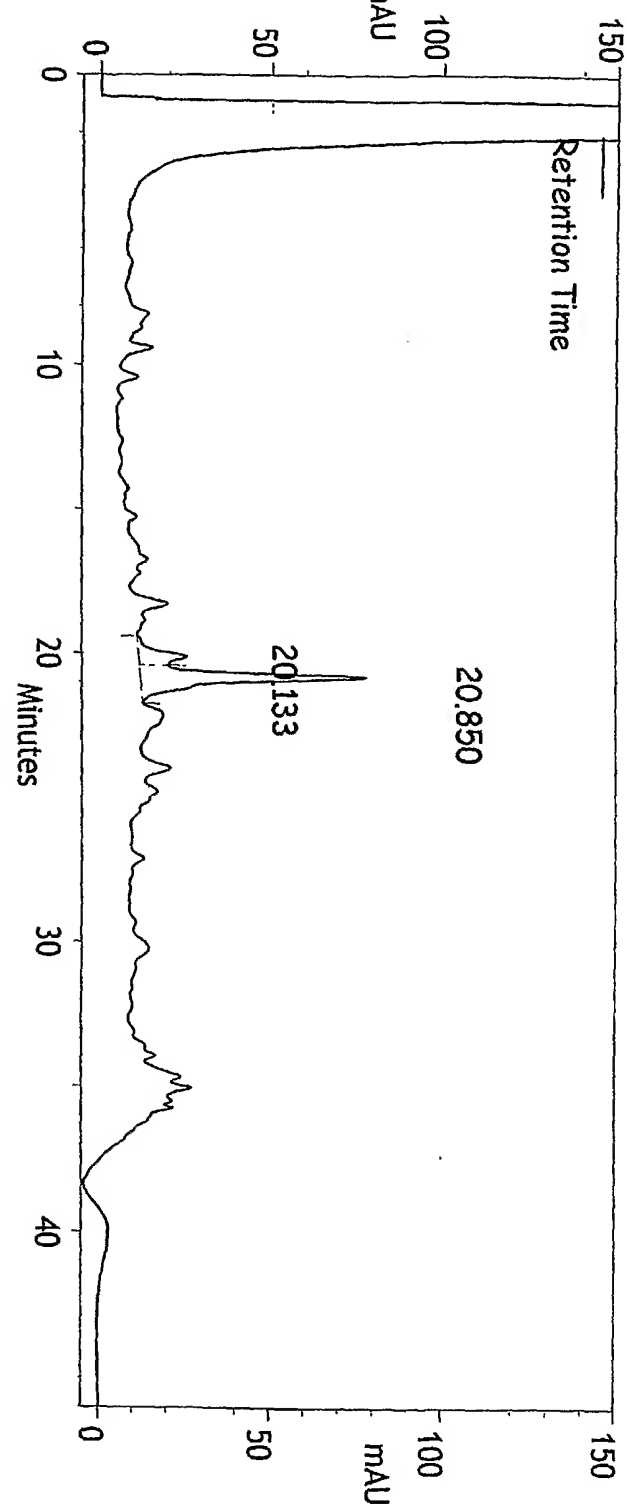
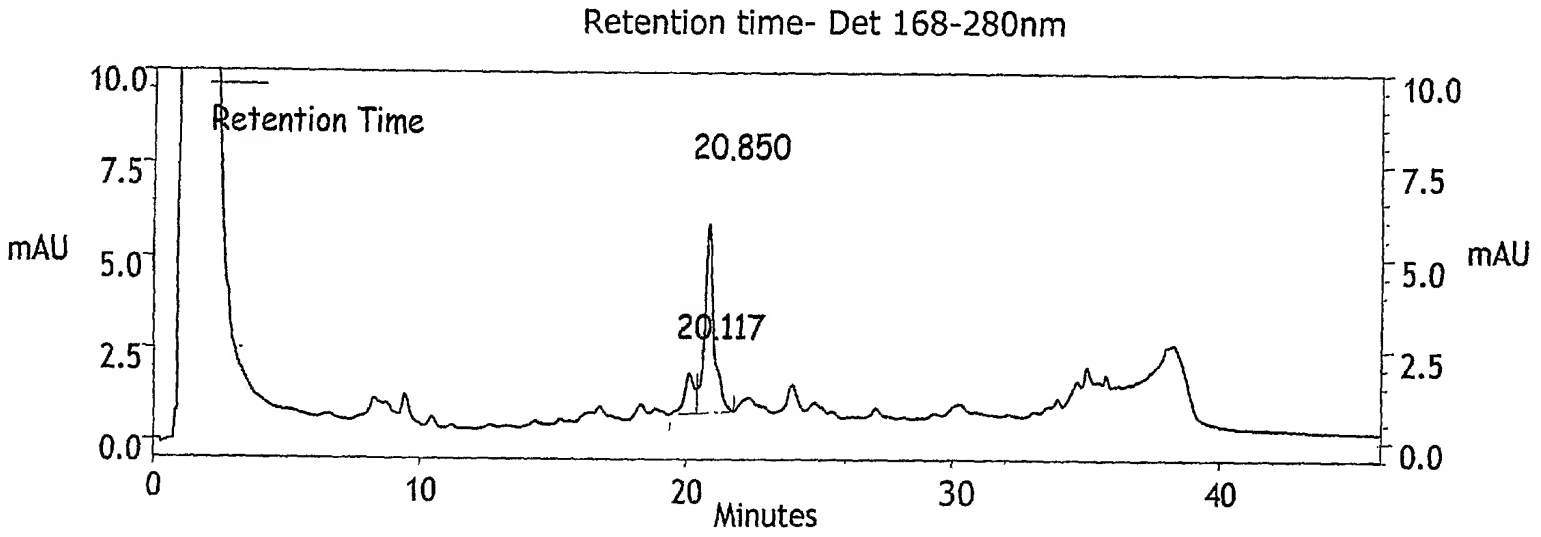


Fig. 4

Retention Time	Area	Area%	Height	Height %
20.133	366620	18.96	14015	17.57
20.850	1566761	81.04	65735	82.43
Totals	1933381	100.00	79750	100.00

Fig. 5



Retention Time	Area	Area%	Height	Height %
20.117	28401	18.50	1093	17.56
20.850	125111	81.50	5130	82.44
Totals	153512	100.00	6223	100.00

Calculated [Epo] =44.8 ug/mL

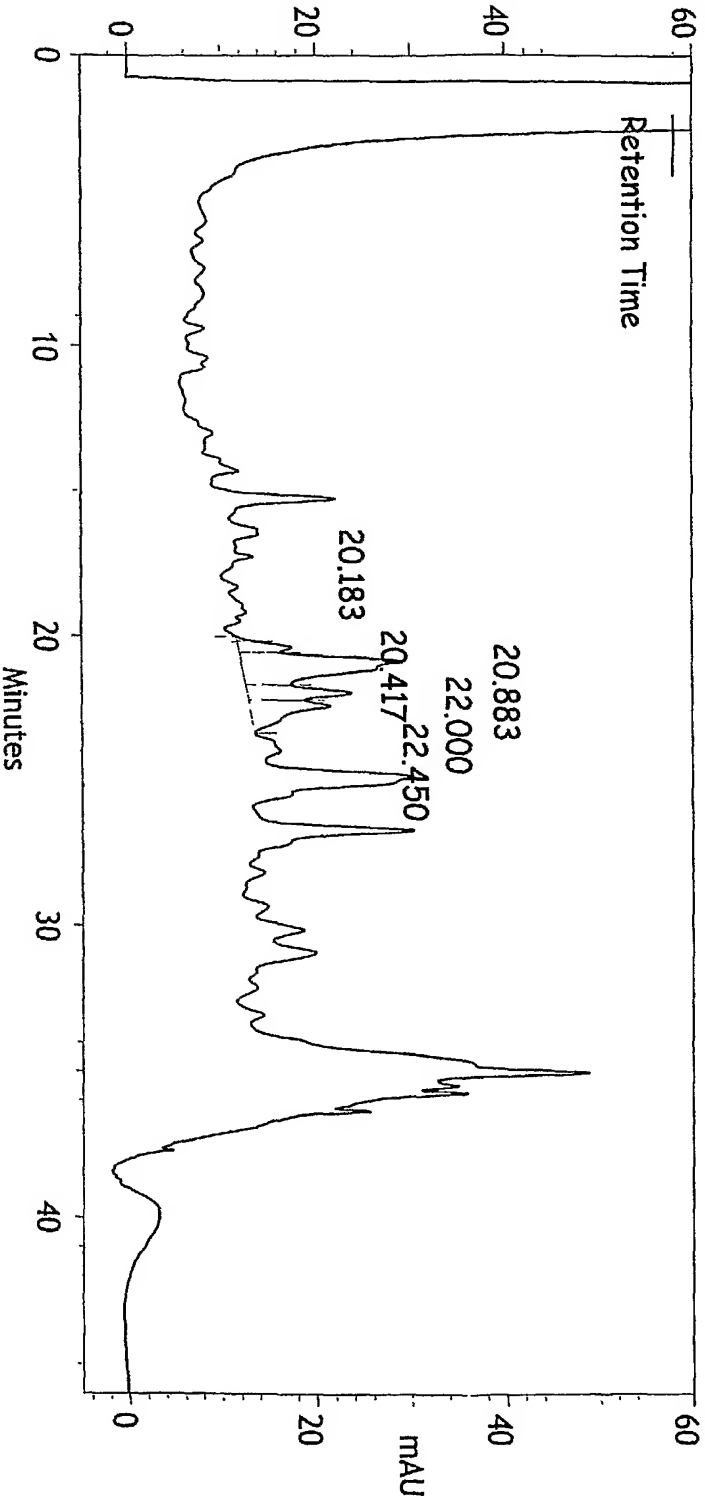
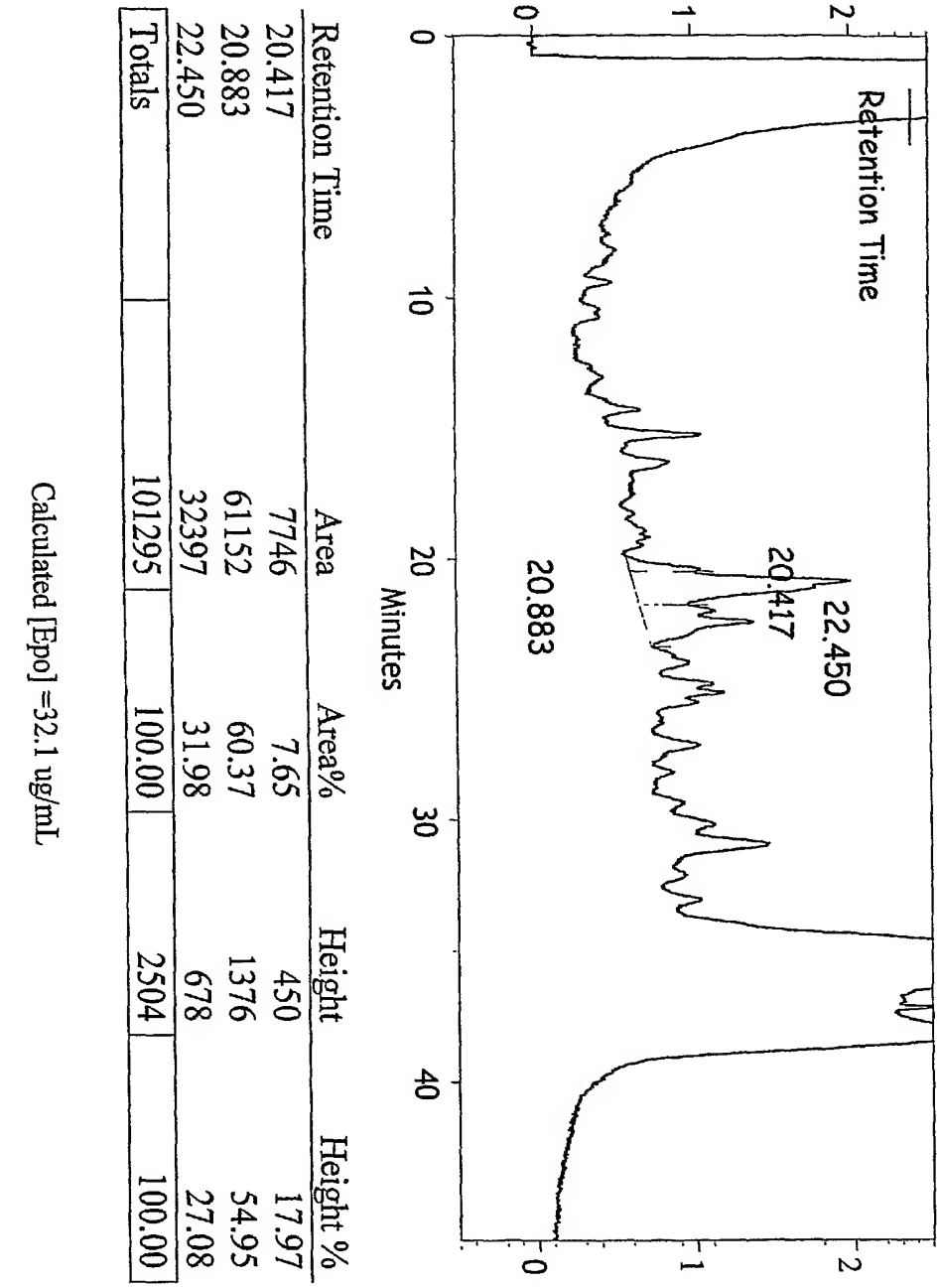


Fig. 6

Retention Time	Area	Area%	Height	Height %
20.183	6204	0.47	1296	3.01
20.417	98943	7.43	5574	12.96
20.883	703365	52.78	16687	38.79
22.000	259538	19.48	10964	25.48
22.450	264509	19.85	8501	19.76
Totals	1332559	100.00	43022	100.00

Fig. 7



Retention Time	Area	Area%	Height	Height %
20.417	7746	7.65	450	17.97
20.883	61152	60.37	1376	54.95
22.450	32397	31.98	678	27.08
Totals	101295	100.00	2504	100.00

Calculated [Epo] =32.1 ug/mL